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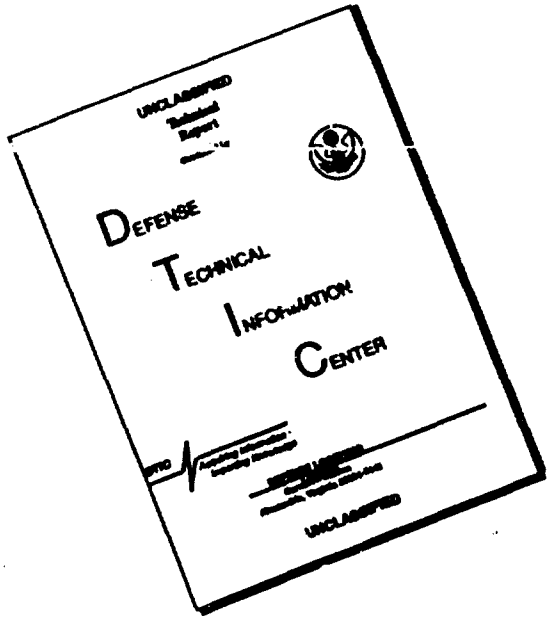

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STUDIES ON VARIATION OF THE RICE BLAST-FUNGUS PIRICULARIA ORYZAE-CAV.

I. Karyological and Genetical Studies on Variation

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Contents

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I. Introduction

Among the diseases of rice plant, the damage due to rice blast is most severe and it is important to cultivate the kind of rice which is resistant to rice blast. The cultivation of rice resistant to rice blast was first conducted by combination between the Japanese rices. Thus, the Agriculture and Forestry No. 22, 23 and many other kinds are cultivated and spread to the entire country (Ito, 1955). However, since the successful crossing of Japanese rice with imported Chinese rice by Iwaki (1942) which resulted in the cultivation of highly resistant Jinzyu and Hutaba rice, the attempts to cultivate highly resistant rice by crossing Japanese rice with foreign rice have overcome many difficulties. (Kovama 1952, Hamura, Kitamura 1954, Shihara 1955, Shihara, Danabe 1959, Ito et al 1961, Kitamura 1962). Recently, the phenomena of suddenly losing these resistant properties and becoming suceptible to diseases appeared at various places. In 1952, the resistant properties of the hybrid between Northern Chinese rice and Jinzyn rice decreased to the extent of Aichigyoku rice according to the experiment at Aichiken Agricultural Experimental Station. And also Kanto No. 51-55 have similarly decreased the resistant properties. (Shihara and Nakanishi 1959). Kanto No. 54 which was cultivated at Agricultural Station of Shitaima, Naganoken in 1953 had many rice blasts.(Chiku 1956). One of the causes for these is considered to be the biological differentiation of Piricularia orvzae. Since 1955, a joint research on race of Piricularia orvzae was conducted and an idiosyncratic reaction between the type of rice and fungus race was clarified. Thus, the method of judging race is established and the anual rise and fall of race distribution is clarified (Goto et al 1961). However, the cause and mechanism of the race differentiation is not widely investigated and the adequate explanations are

lacking. The authors attempted to analyze from the karyological and genetical standpoint in order to obtain basic views on variation of rice blast fungus. The researches are just beginning and many problems remain to be solved. Here, the results obtained thus far are reported.

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II Past Research

In our country, Sasaki (1922, 1923, 1925) has first reported the biological differentiation of rice blast fungus. The causes of diseases are classified into three classes of A, B and C from the properties of rice gel culture at Aichiken, and A and C are similar whereas B is remarkabley strong. Later, Nozu (1928) reported that another classification of disease in addition to the above classifications was found at Shimaneken. Since 1931, a systematic research has begun at Agriculture and Forestry Department to eliminate and prevent the rice blast disease. The researches in this field were also actively conducted at Kyoto and Hokkaido Universities and the biological differentiation phenomena of rice blast disease were investigated from various angles.

For attempts on classification based on culture properties, the following researches were conducted. Tochinai and Shimamura (1932), Shimamura (1932) have cultured 341 stocks of bacilli from infected plants collected at various areas of Japan on four different culture media and classified into 9 types based on their culture properties. Itsumi et al (1936) compared the growth type in four different culture media using 33 systems and classified into 9 groups from the status of mycelial growth

or color, formation of condium and color adsorption on culture media. Aoki (1935) classified into 14 types according to mycelial formation in air on 1 % sucrose added potato gel, conidium formation, the color adsorption of mycelia and mycelial growth in synthetic culture media with different amount of sugar using 23 systems. Ohtsuka (1961) has bred rice blast fungi in 12 kinds of culture media using 45 systems and observed the growth status, the state of mycelia, adhesion of conidium and pigment production. He has made clear that no relationship exists between the difference in the various culture characteristics and strength in the causes of diseases same as the previous researches.

As classifications according to culture temperature, Tochinai and Shimamura (1932) and Shimamura (1932) classified 5 types which grow better at 28°C than at 25°C and 4 type which contrasts above. Konishi (1932, 1933) showed that all systems grow better at 28°C and classified into 3 growth types at 32°C. Sumimoto and Kano (1961) have cultured the flat ground fungi and high ground fungi on 2 % sugar added gel culture media for 3 years from 1955 to 1958 and classified into three groups, one growing better at 31°C than 14°C, one growing better at low temperature and one indistinguishable between the two.

As the classification according to the properties, the following researches were conducted. Tochinai and Shimamura (1932) measured the length of conidium on rice plant and classified into four systems of a large spore forming type with an acute tip and a small spore forming type with an obtuse head. Tomikata (1961) reported that the conversion into single spore from three spores by NH_4 salt treatment of a durable spore is different depending on the system. Suzuki (1953) has classified 12 types of rice blast fungi according to the germination type of conidium, adhesion forming type, the shape of adhesion apparatus and the existence of adhesion apparatus formation. Huchikawa et al (1954) confirmed that the temperature range for the formation of adhesion apparatus is different depending on the system. The classifications according to biochemical properties have been done earlier. Itsumi et al (1936) and Ikeya (1934) have cultured 29 systems at 24-25°C for 4-6 weeks on 1 % sugar added potato liquid culture media and classified into three groups according to the pH changes of filtered liquid. The relationship between poisonous properties and strength of disease was not confirmed. Ito (1938, 1939) and Itsumi et al (1941) cultured 21 systems on synthetic culture media made by adding regenerated cellulose precipitate from filter paper and compared the separation by cellulose. The separation by cellulose was clear cut and three groups of strong, medium and weak could be classified. These separations are parallel to disease separation and system with a large degree of separation had a strong

disease.

Ohtani (1958 a) examined a few systems with different diseases and made the following facts clear. The maximum length of fungi attained in three weeks of culture on synthetic media is not much different but the growth speed is faster for fungi of strong disease. The strength of breath is stronger for strong disease and the maximum values appear at an early period of culture. The activity of amino acid oxidizing enzyme is higher for strong fungi and appears at an early stage of culture. There was no relationship between the activity of enzyme and the strength of disease. No difference exists between the system in producing poison.

Employing 47 systems, Ohtsuka (1961) classified into three types according to the demands of diamine, nicotinic acid, biotin, $NaNO_2$, Inurin, Solvose and tryptophan and reduction of mitric acid as a result of experiment performed on culture and biochemical properties, vitamin and amino acid formation, enzyme activity and infrared abosorption spectrum, and indicated that no relationship exists between classifications according to the biochemical differences and strength of diseases, Yamagaki and Toya (1956) and Yamazaki (1961) investigated the differences in $CuSO_4$ resistant properties of various rice blast fungi from all over the country and the growth limit concentration by culturing on $CuSO_4$ added potato gel culture media at various concentrations and clarified the variations from 12 m mol to 28 m mol. Chiba et al (1958) confirmed that no difference in reagent resistance between 22 bacilli stocks separated at Aomoriken exists.

Itsumi (1934, 1949) has limited the biological differentiation phenomenon of rice blast fungi to the parasite and disease and classified the biological type based solely on the relationship of parasite and disease bacilli.. When the other biological classifications coinciding with the disease differentiation were discovered, he proposed that it is safe to judge on the biological type and made an effort to clarify the disease differentiation. Itsumi et al (1934, 1941), abe (1936) and Izyo (1936) have classified into 11 groups based on the strength of disease and inoculation experiment on sapling and neck of rice. The disease against sapling was weak and against neck was strong. And there was a system whose properties are contrast with above. Since 1955, the agricultural experimental station of Hokkaido, Nagano, Aichi, Kihu and Department of Pathology, Agricultural Technical Research Laboratory have launched a cooperative research efforts and established the method of judging the race of rice blast fungi (Goto 1956, 1963, Goto et al, 1961, Goto and Yamanaka 1956, Iwata and Narita 1956, Kuribayashi 1959,

Nakanishi and Imamura 1956, Nahanishi and Uzihara 1956, Yamanaka 1957). For example,the selected twelve kinds, Te-tep, Tadukan, Karasusaki, Chyokonae, Notoriben, Kanto No. 51, Ishikarihakumo, Homarehishiki, Ginga, Norin No. 22, Aichigyoku and Norin No. 20 are largely classified into T group penetrating the foreign rice, C group penetrating Chinese rice excluding other foreign rice and J group penetrating only Japanese rice, and each group is further classified into 14 races. From the results of differentiating about 900 bacilli stocks in 1960 and 1961, it was made clear that Ishikarihakumo B, two races of other Japanese kind N-2 of S and N-1 of S are abundant in entire country. Its distribution has a regional property and C-1, C-2 were large whereas T group race was small. The similar results are independently obtained by Ohya (1958, 1959) and Chiba et al (1958) at the same time. Employing Piricularia oryzae from India, Ohya (1958, 1959) showed from inoculation experiment that it is entirely different from Japanese race. Further, the research on the race is extended to the race of foreign product. Takasaka et al (1964) inoculated 12 bacilli stocks to Japanese kinds and showed that Japanese type race and Southern type race both have started to distribute in Formosa. Also the research on race is conducted in the United States (Latterell et al 1954, 1960) and Formosa (Hung et al, 1961).

Although it was established that differentiation in the properties of each kind is taking place, the causes for the differentiation i.e. the mechanism of variation were rarely investigated. Konishi (1933) reported the appearance of sector when No. 18 bacillium from single conidium was cultured on 1 % sugar added potato gel culture media at 35°C. This has the entirely different properties from mother bacilli and their properties are maintained by transplantation of single spore. Shimamura (1932) also reported the appearance of sector when gingko gel and synthetic gel culture media are used. Kuribayashi (1953) observed two cases of variations in disease. One is due to sectoring and 19 % of 210 strains from Naganoken produce brown or grey sector in black homogeneous colony of disease were different from parantal mycelium. The other case is due to mixed inoculation of two strains of spore. Three groups among five were stronger than parantal mycelium and two groups were weak. From these results, Kuribayashi thought that Piricula a oryzae forms a sector in a single spore culture and it is the esult of hybrid, not a sudden variation from frequency of their appearances. And it is considered the variation can occur as a result of hybrid due to adhesion of mycelia. Goto and Yamanaka (1958) and Yamanaka (1963) observed the sorting out of bacilli strain which indicates a strong disease in Chinese rice or its hybrid or highly resistant Tadukan hybrid. Nakanishi and Imamura (1960) stated that there are sometimes diseases in conidium formed by

single disease spot and the different bacilli strains from the parental strains might be sorted out. Goto and Yamanaka (1960) obtained bacilli different from parental system as a result of mixed inoculation on two receptive systems and investigation of 74 bacilli from a single spore and concluded that these variants were formed by the mixture of parental system and combination of bacilli or a sudden variation. Suzuki (1960, 1962) found following three types as the cause of bacillie loss by culturing. 1) Spore and mycelium are not formed. 2) Only mycelium is formed. 3) Mycelia and spore are formed but the adhesion apparatus is not formed and bacilli are lost. They have discussed that these bacilli form wild type and heterokaryon and play an important role in maintaining the race and strength of disease. Shitayama et al (1964) found three kinds, sudden loss of bacilli during culture, sorting out from particular bacilli strain and gradual bacilli loss during culture, and showed the type which does not form adhesion apparatus and a peculiar shape with bad bud and short and fat budding tube having branches as a result of investigating the spore germination of two previous bacilli strains and adhesion apparatus formation. Goto and Tamada (1964) discussed on the ramble variation of bacilli and conidium formation employing Piricularia oryzae of single spore separation system. Kosagahara (1959) obtained the variant strains (maximum 20 %) having normal growth and spore formation but low germination rate and found that lactic acid contents are low in spore and when a small amount of lactic acid is given, the germination rate is recovered almost 100 %.

Experiments on inducing the variation artificially are very few. Yamazaki and Tova (1951, 1957) reported that many sectors appear and $CuSO_4$ resistant bacilli are obtained when bacilli are cultured in $CuSO_4$ potato gel media. Yamazaki and Tobo (1953) obtained the biochemical sudden variant concerning the carbon metabolism in similarly obtained sector. Tomizawa (1953, 1956) obtained the adenine requiring system by ultra violet light. Kuribayashi et al (1955) obtained the shape variant by ultra violet light, and inoculation experiment and investigation of various culture properties are conducted. Yamazaki and Murada (1963) and Yamazaki et al (1964) obtained many biochemical and shape sudden variants and investigated the changes of bacilli in detail. They have also discussed the relationship between nutritional requirement and bacilli.

III Karyological Studies on the Living Environment

A. Introduction

Piricularia oryzae is susceptible to variation in natural state and during the culture. Also, the biological and

shape variations take place easily in single-spore isolates. Condium of rice blast fungus generally consists of three cells, and each cell germinates. Therefore, it is an important problem in pursuing the variation of the single-spore isolates to clarify whether the nucleus in each cell is hereditary or not. As a first step, it is very important to study not only nuclear division in the process of conidium formation but also nuclear activity through living environment in addition to the clarification on the number of nuclei in each part.

Karyological studies on rice blast fungus were already conducted by Yamazaki (1953a, 1953b) and Suzuki (1953a, 1953b, 1953c, 1955, 1963). Suzuki has asserted the following, Mycelium, spore, conidophore, and each cell of adhesion apparatus contain many nuclei and the phenomenon of heterokaryosis can be seen. Also in the single spore isolates, almost all of them are heterokaryon, In contrast to the above, Yamazaki stated that one cell principally contain one nucleus through the living environment. Mizuzawa (1959) observed conidium of rice blast fungus through electron microscope and showed clearly an existance of one nuclei in one cell through the pictures.

It is, therefore, necessary to make clear whether these contradicting results are due to the difference in bacilli employed in experiment or the difference in nuclei staining technique. Employing many different systems and several types nuclei staining method, we have observed the nuclear activity in detail and nuclear division through the living environment. Our results are in agreement with one cell-one nucleus principle and the details are reported here.

B. Materials and Method;

Bacilli

We have employed 22 single spore isolates of wild type (include bacilli other than rice blast fungi) which was preserved by paraphin flow preservation method, 9 systems of P-1, P-2, P-2b, Ken 53-33, Ine 72, Kita 1, Ken 54-20, Ken 54-04 and Ine 168 obtained from Pathology Department, Agricultural Technology Research Laboratory and three systems of No. 5, No. 11 and No. 116 obtained from Prof. Suzuki of Tokyo Agricultural Engineering School. Total 34 system. Unless otherwise noted, P-2 bacilli which have a good conidium formation are used in each experiment.

Method of cytological observation

Method of making preparato (transliteration); In order

to make preparato(transliteration) for observing nuclei during condium formation process and in stationary state of conidium, 7-10 days culture are done on potato gel or agar media at 28°C. The cover glass is lightly pressed on the colony and taken off after 2-3 minutes. Then, spore and mycelia adhere to the cover glass. If albumin is lightly pasted on cover glass and dried under alcohol lamp, the adhesion is better. The appropriate materials for observing the nuclei activity at each period during conidium formation process from conidophore are thus obtained.

In order to make preparato (transliteration) for observing nuclei of germinated spore or mycelium, the culture is carried out at 28°C by floating the spore over potato gel liquid or rice plant gel liquid on slide glass. At appropriate period, albumin pasted cover glass and a few filter paper placed over glass are lightly pressed. At this time, it is desirable to have culture liquid spread on the entire area of cover glass. When these slide glass and cover glass are placed in fixed liquid, the spore and mycelium adhere to cover glass.
In making the materials for adhesion apparatus, water is used instead of culture liquid according to the above method. The adhesion apparatus sticks well on slide glass and can be used as preparato (transliteration) after discarding the water.

Nuclear staining method; The nuclei were stained by the following several method using cover glass (in case of the adhesion apparatus, slide glass) prepared by the above method.

HCl-Giemsa Method; A simplified method of Ribinow (1944) and Hrushovetz(1956) was employed. After three parts of alcohol and one part of glacial acetic acid are settled for 10-15 minutes, 95 % alcohol was passed through for 5-10 minutes and 70 % alcohol for more than 30 minutes. Water is changed two to three times and washed for about 5 minutes. Then it is hydrolyzed by IN HCl (60°C) for 7-8 minutes. After washing with water (5 minutes) it is stained with Giemsa color solution for 30 minutes to two hours. Giemsa color solution was made by diluting the commercial Giemsa liquid with Sörensen buffer solution (pH 6.9-7.0). After the staining, the excess dyes were removed by washing with water for 1-2 minutes and 10 % glycerine was placed. The cover glass and slide glass overnight for a good adhesion. These can be preserved for long period of time.

Staining by basic fuchsin

Although Feulgen's nuclear dyeing method gives good results, DeLamater (1948) method is simple and gives better results. This method is improved and dyed for shorter time by

the following simplified method. The hydrolysis by IN HCl is same as HCl-Giemsa method. After treating with 2 % formalin water for 2-4 minutes and washing with water, the dyeing is carried out for 15-30 minutes with 0.5 % basic fuchsin solution. After dyeing, it is washed with water and passed through alcohol and kishroll. Then it is sealed with balsam.

Carmine acetate and Orsein acetate dyeing

After three parts of pure alcohol and one part of glacial acetic acid is settled for 1-3 hours, it is washed with 95 % alcohol for 5-10 minutes. It is treated with IN HCl (room temperature) for 5 minutes and then hydrolyzed with IN HCl for 7-8 minutes at 60°C. After washing with water, it is dyed with orsein acetate or carmine acetate. In the case of carmine acetate, it is dyed with mordant using 4 % iron-alum water after washing. Good results are obtained if the dyeing solution is heated to temperature below its boiling point by alcohol lamp.

Observation of nuclei in living body (Microscopic observation method of phase difference) ; Three percent of agaragar or 10 % gelatine culture media are dissolved and cover glass (24x24 mm) is dipped into these and dried to form a thin agaragar or gelatin film. Agaragar or gelatin are removed from one side of the cover glass and only the part of edges on the other side. Then, this film is lightly pressed on the colony of rice blast fungi from a separate culture to adhere spore or mycelia. A separate cover glass is closely adhered on the above film and agaragar film sandwich is made with two cover glasses. If the edges of the surrounding part between two cover glasses where there is no agaragar film are sealed with fluid paraffin, the agar film is prevented from drying and convenient to observe longer period of time. These cover glasses are placed over slide glass with a hole in the center and observed microscopically. In microscopic observation, Japanese optical apparatus for phase difference was employed. The observation is done in dark medium 100 x and also the pictures were taken.

C. Results

1. Comparison of nuclear dyeing method

According to Robinow (1944), Hrushvetz (1956), Krox-Davies and Dickson (1960), Ward and Ciurysek (1960, 1961), the good results were obtained in nuclear dyeing of bacteria and mould by HCl-Giemsa method. This method also gives very good results in dyeing rice blast fungi, Orsein acetate and

carmine acetate are suitable for observation since the dveing objects appear to expand their shapes. However, orcein acetate does not seem to dve nuclei in stationary state. Giemsa method is very effective in nuclear dveing, but the particles other than nuclei in the cell are also dved by the longer time of dveing. The basic fuchsin method gives also a very good result. Hematoxsvrin dveing is effective in selective nuclear dyeing of conidium in stationary states but it often dyes particles other than nuclei in mycelia or germinated spore. Therefore, this method is not considered to be a suitable method for nuclear dyeing. Dveing by Azur A has been attempted but failed to dve nuclei of conidium and mycelia.

2. Number of nuclei in conidium and nuclear division.

Conidium formed on rice plant or culture medium consists of three cells (Figure plate 1-A, 1) and rarely of 2 or 4 cells (Figure plate 1-A, 2). Each cell contains one nucleus (Table 1, Figure plate 1-A, 1, 2). Microscopic observation of living body confirms that one cell contains only one nucleus (Figure plate 1-A, 3). Piricularia species penetrating plants other than rice such as millet (s-156-1), barn yard millet (H-80), mioga (2-187), mehishiba (G-235) and broom-corn (p-168) had one nucleus in one cell. In table 1, there are few cells with two nuclei but the rate is extremely low. It is noticed that CuSO4 resistant bacilli Cu-633 derived from P-2 had 10 % of cells with 2-3 nuclei.

Table 1. Number of nuclei in conidial cell

Strain	Number of nuclei in each of conidial cells**						Total	Percentage of conidia each cell of which contains one nucleus
	1+1+1	2+1+1	1+2+1	1+1+2	2+3+2	1+1+1+1		
P-2 b	305	1	0	0	0	0	306	99.6
Ken 53-33	229	0	0	0	0	0	229	100.0
Ina 72	69	0	0	0	0	0	69	100.0
Hoky 1	252	0	1	0	0	0	253	99.6
Ken 54-20	322	1	0	0	0	0	323	99.7
Ken 54-04	271	0	0	0	0	0	271	100.0
Ina 168	226	0	0	1	0	0	227	99.6
P-1	317	4	1	0	0	0	332	98.4
P-2	320	2	0	0	0	2	324	98.7
Cu-633*	321	21	5	1	1	5	354	90.7

*: Cu-633 is $CuSO_4$-resistant strain derived from P-2

**: The numerals indicate in this order the number of nuclei contained in apical-, central- and basal-cell of conidia.

When the conidium formed on rice plant or culture medium is placed in water or culture solution, it starts to germinate after few hours. The base germination tube is mainly formed in front or base part of the cell and raely in center of cell. Nucleus in conidial cell is directly migrated to germination tube (figure plate 1-A, 4a, 4b) for one case. For the other case, the nucleus in conidial cell is divided into two and only one nucleus is migrated into germination tube leaving the other nucleus in conidial cell (figure plate 1-A, 5a, 5b). The frequency of the above occurance is as shown in Table 2, and the frequency of occurance on the latter is larger. During the migration of nucleus from conidium to germination tube, the nucleus takes the elongated form (figure plate 1-A, 4a,5a).The remaining nucleus in conidium does not divide

Table 2. Number of nuclei in conidial cell at one cell stage of germ tube (P-2)

Type	No. of conidia observed	Frequency (%)	Remarks
	50	77	One nucleus moved into germ tube after nuclear division
	15	23	One nucleus moved into germ tube without nuclear division
Total	65	100	

Observation was made 5 hours after incubation in the rice straw decoction medium.

Table 3. Number of nuclei in conidial cell after germination (P-2)

No. of nuclei in each of conidial cell			No. of conidia observed		Frequency (%)
Apical-cell	Central-cell	Basal-cell			
0	1	1	8		
0	1	2	1		
0	2	1	5		
0	2	2	4		
0	2	3	1		
0	3	1	1	53	19.8
0	3	2	1		
0	1	0	1		
0	2	0	3		
1	1	0	9		
1	2	0	18		
2	2	0	1		
1	1	1	61		22.8
1	1	2	12		
1	2	1	81		30.3
2	1	1	3		
1	2	2	43		16.1
2	2	1	2		
2	2	2	2		
1	3	1	4		
1	3	2	3		
2	3	2	1		
1	2	2	2		
Total			267		

Observation was made 24 hours after incubation in the rice straw decoction medium.

further but in some cases, divide 1-3 times into 2-8 nuclei (figure plate 1-A, 6,7). In case of two germination tubes from one cell, one of the divided nuclei first goes into germination tube and the other nucleus remain in conidium or goes into the other germination tube. Also the remaining nucleus in conidium can be further divided and one nucleus migrates into germination tube. In case of forming 3 germination tubes from one cell, the process is the same as above. Table 3 shows an example of nuclei distribution in germination spore after 24 hours of culture. The direct migration of nucleus in conidial cell with nuclear division was 53 among 267 and amounts to 20 %. This result is almost similar to the result of table 2. The number of nuclei in front, center and base part of the cell is 1·2·1 (30.3 %) which is the most abundant and next 1·1·1(22.8 %), 1·2·2(16.1 %). In case of 1·2.1, each cell is divided once and front and base part of the cell is germinated with a nucleus migration.

Table 4. Relationship between germination of conidia and their nuclear division (P-2)

Type	No. of conidia observed	Frequency (%)	Remarks
A	53	11.0	Nucleus moves into germ tube without division.
B	327	67.6	One nucleus moves into germ tube after one nuclear division.
C	3	0.6	Two nuclei move into germ tubes after one nuclear division.
D	66	13.6	One nucleus moves into germ tube after one nuclear division, and the remaining one divides again into two nuclei.
E	20	4.1	One nucleus moves into one of germ tubes after one nuclear division, and the remaining one divides again and then one nucleus moves into the other germ tube leaving the last one in conidial cell.
F	1	0.2	Three nuclei produced by two nuclear division move seperately into each of three germ tubes.
G	1	0.2	One nucleus moves into germ tube after two nuclear division, and the other three nuclei remain in conidial cell.
H	9	1.9	Two nuclei move separately into each of two germ tubes after two nuclear division, and the other two remain in conidial cell.
I	4	0.8	Three nuclei move separately into each of three germ tubes after two nuclear division, and the last one remains in conidial cell.
Total	484	100.0	

Observation was made 24 hours after incubation in rice straw decoction medium.

In case of 1·1·1, the front and base part of cell is divided once and a nucleus is migrated into germination tube. The center cell is considered not to produce germination tube nor nuclear division. When the relationships of nuclear division and germination concerning the cell produced germination cell are considered from the data of table 3, they are as shown in table 4. When one germination tube is produced, the nucleus migrates into germination tube without nuclear division (type A). The nucleus divides once into two and one nucleus migrates into germination tube leaving the other one in conidial cell.(type B). The nucleus remaining in conidial cell is divided into two (type D). The nucleus remaining in conidial cell is divided into three and one of the four nuclei formed by two divisions and a germination migrates into a germination tube and the remaining three are in conidial cell (type G). When germination tubes are more than two, the nuclei become 2-4 by 1-2 divisions and each nucleus migrates into germination tube without remaining in conidial cell, (type C,F) or 102 nuclei remain in conidial cell (type E,H,I). From the above observation, it is clear that nuclei in conidial cell do not distribute but one nucleus divides 1-3 times and only one nucleus among them migrates into the germination tube.

3. Number of nuclei and nuclear division in mycelium and conidophore.

Although the nuclei in mycelia show various shapes, one cell generally contains one nucleus (figure plate 1-B,8,9). There are cases of two nuclei in one cell rarely but these are considered to occur right after division. The results of investigating the number of nuclei in mycelial cell are shown in table 5. Most of the cells contains one nucleus but some contain 2-6 nuclei. The proportion of cells containing more than two nuclei is different depending on the system and 1.1 %-16.8 % variations are shown. However, the average number of nuclei per cell is 1.01-1.20, thus confirming the one cell-one nucleus principle. Saka-1, Saka-2 and Saka-3 which have relatively high ratio of multinuclei cell are peculiar systems with tendency of producing sectors and variations in potato agar culture. Piricularia species isolated from plants other than rice have one nuclei in one cell as shown in table 5. Suzuki (1963) reported on three systems of No. 5, No. 11 and No. 116 indicating that the number of nuclei in a mycerial cell is 4 in No. 5 with distribution of 1-11. Similarly No. 11 is 5 (1-10) and No. 116 5(1-13). As shown in table 5, we have observed only one nucleus in one cell and 2 or 3 nuclei in a cell were very rare.

In mycelium of conidophore, one cell contains one nuclei

(figure plate 1-B, 10). In case of conidium formation, the nucleus formed by division at front cell of conidophore migrates into newly formed conidium.

4. Nuclear division during the process of conidium formation.

When the tip of conidophore begins to swell, one nucleus migrates into it. Conidium is initially spherical in shape (figure plate 1-B, 11) but changes to fusiform gradually. (figure plate 1-B, 12a, 12b). As is clear from the diagram, one cell of conidium contains one nucleus, but by nuclear division, it becomes two (figure plate 1-B, 13a). When the nuclei migrate to both end (figure plate 1-B, 13b), a membrane is formed at the middle and separation into two cells takes place. (figure plate 1-B, 14). One of these nuclei is again divided (figure plate 1-C, 15) and third conidial cell with one nucleus is formed. From the above observation, the nuclei in three cells were originated from a single nucleus and considered genetically the same. During transformation of two cells into three cells, it is not often observed which nuclei are easier to be divided, but apical cells divide in many cases. There are cases where both nuclei divide, but these are considered to be spore (figure plate 1-A, 2) having 4 cells.

Table 5. Number of nuclei in mycelial cell

Strain	Host	No. of nuclei in mycerial cell						No. of cells observed	No. of multi-nucleate cells	Frequency of multi-nucleate cells (%)	Average no. of nuclei per cell
		1	2	3	4	5	6				
Hoku 1	O. sativa	515	6					521	6	1.1	1.01
No. 11		533	7					540	7	1.3	1.01
P-2		994	18					1012	18	1.7	1.02
No. 116		523	10					533	10	1.8	1.02
15-1		519	10					529	10	1.9	1.02
Ina 168		281	6					287	6	2.1	1.02
4-2		497	11					508	11	2.1	1.02
P-1		1043	25					1068	25	2.3	1.02
56		520	13					533	13	2.4	1.02
18-3		523	14					537	14	2.6	1.03
42-1		508	11	3				522	14	2.6	1.03
7-1		510	15	1				526	16	3.0	1.03
6		500	16	1				517	17	3.2	1.03
No. 5		520	23	1				544	24	4.4	1.05
50		501	20	2	2			525	24	4.5	1.06
19		516	24	1				541	25	4.6	1.05
Saka-1		1827	207	25	9	2	2	2072	245	11.6	1.15
Saka-2		507	61	13	4			587	78	13.2	1.17
Saka-3		429	75	5	7			516	87	16.8	1.20
H-80	P. crus-galli	513	8					521	8	1.5	1.02
G-59-2	D. adscendens	566	10					576	10	1.7	1.02
Z-6	Z. Mioga	568	16					584	16	2.7	1.03
S-79-1	S. italica	512	22					534	22	4.1	1.04
P-168	P. miliaceum	537	26					563	26	4.6	1.05

Table 6. Nuclear division at two cell stage of conidia

Type	No. of conidia observed	Frequency (%)
Nuclear division in apical-cell	21	80.8
Nuclear division in basal-cell	2	7.7
Nuclear division in both cells	3	11.5
Total	26	100.0

5. Nuclei in appressoria

Number of nuclei in appressoria formed on slide glass was observed at 24 and 45 hours after incubation. As shown in table 7, the cell having one nucleus is the largest and few cells having two nucleus appear after 24 hours of incubation. After 45 hours of incubation, cells having maximum 6 nuclei appeared. Since the mycerial formation on slide glass can not be done, nuclear division is continued in appressoria and the number of nuclei are increased with the time. The number of nuclei in appressoria formed on rice plant is not clear but in case of mycerial formation, one nucleus among two nuclei formed by the division in appressoria is considered to be migrated.

6. Mode of nuclear division

The mode of nuclear division seems to be different in conidium and mycelium. In condium, germination starts after few hours in water or culture solution, but the nuclear division starts after the germination in one case and before commencement of germination in the other case. The conidial nucleus in stationary state is considered to be dveable clot but the commencement of nuclear division loosens the clot and forms the different size of dved object (figure plate 1-C, 17,18). The dveing object forms a typical equatorial plate in the middle period (figure plate 1-C, 19) and through the latter period (figure plate 1-C, 20, 21), it goes into the final period (figure plate 1-C, 22, 23). The number of dveing objects are easier to observe at the beginning of latter period, and three are observed in one case and 5-6 are observed in another. (figure plate I-D, 24, 25). This may be due to the difference between single nucleus and double nuclei. At any rate, the number of stained object should be further investigated.

The nuclear division of mycelial cell (specially significant in atmospheric mycelia) is different from conidium and a typical fibrillar division was not observed. Initially, the dye clot in stationary state melts and forms in belt or spherical types (figure plate I-D, 26). These are vertically divided from one end to the other end (figure plate I-D, 27, 28). At this time, the division is always perpendicular to the direction of mycelial growth. The dved objects connected to belt or spherical shape form two vertical rows and one row migrates into the direction of mycelial growth. (figure plate I-D, 29,30,31). Thus the nucleus divides into two.

It is considered from the above observations that the nuclear division of conidium is fibrillar division similar to high organisms. The nuclear division of mycelium is considerably

different from these. Thus, the detailed future investigation would provide many informations on the subject.

Table 7. Number of nuclei in appressoria formed on slide glass

No. of nuclei in appressoria	Hours of incubation	
	24	45
0	88	18
1	69	128
d	31	24
2	5	37
1+d		8
3		13
2+d		2
4		12
3+d		1
5		2
6		1
Total	193	246

Observation was made 24 and 45 hours after incubation in the rice straw decoction medium.
d: Dividing nucleus

D. Discussion

Piricularia species are very susceptible to variations. In order to understand the mechanism of variations, it is important to know the nuclear activity through the living environment. In bacilli, there are generally single spore isolates and single cell isolates (by the division of mycelial cell). Many types in addition to the above were used in experiments. For the case of a single spore isolates, it is not certain whether they can be considered to be genetically pure. A single nucleus in a single cell can be considered as gentically pure but for multicellular conidium, the single spore isolates can not be used as the genetical research materials unless the nucleus in each cell has been established as genetically pure. There are many conidia and mycelial cells containing multinuclei. If these were mixtures of genetically different species, the variations can occur easily in the single spore isolates.

Nuclear activity during the process of conidium formation is investigated from these view points. The apical conidophore is expanded and conidium formation begins to take place. One nucleus is migrated first and divided into two nuclei. Two conidial cells are thus formed by the membrane separation between the two. One of these nuclei is again divided into two

and the third conidial cell with a single nucleus is formed. Therefore, three nuclei in these conidia were originated from the same nucleus and considered genetically the same. These can be considered the genetically pure single spore isolates. From these view points, the rice disease (Goto, 1954) which contains multinuclear mycelial cells, multicellular conidium, and multinuclei is an entirely different kind of single spore isolates.

Suzuki(1953a, b, c, 1963) and Suzuki et al (1955) reported multinuclear cell in conidium, germination tube, appressoria, mycelium and conidophore. They have made karyological studies and analysis of appressoria and reported that heterokaryosis plays an important role as the causes of variations. These results do not agree with those of Yamazaki (1953a, b). Mizuzawa (1959) and ours. In order to explain these discrepancies, we have investigated the number of nuclei in mycelial cell which was reported as hetero type by Suzuki and obtained the result of one nucleus in one cell similar to other systems. These results are shown in table 5. Therefore, these discrepancies seemed to be the technique involved in nuclear dyeing by Suzuki and not due to the difference in the systems employed in experiments. As will be separately discussed in detail, the multinuclear cells such as Saka-1, Saka-2 and Saka-3 of table 5 have the maximum rate of 17 % and there is no change in the principle of one nucleus in one cell. Suzuki (1963) argued against the researches of Yamazaki and Shinkan (1954, 1959 and 1960) and asserted that "if each cell contains single nucleus, the single spore isolates must be homokaryon, and the variation of biological characteristics would not occur except the confusion of sexual process". Even though variation due to confusion through parasexual generation is not considered, the phenomenon of sudden variation and parasexual recombination was observed as will be described later. This is another support against Suzuki's assertion.

Olive (1953) conducted a thorough literature search and concluded that the nuclear division in cell is via fibrillar division. El-Ani (1956), Knox-Davies and Dickson (1960), Somers et al (1960) and Ward and Ciurysek (1961) indicated a typical fibrillar division in bacilli. Robinow (1956, 1957a,b) and Bakersnigel (1958, 1959a,b,c, 1960a,b, 1961) asserted a separate nuclear division in mycelial cell. Our opinion on these is that fibrillar division is carried out for conidium and separate mode of division is carried out for mycelial cell.

Yamazaki (1953a) reported on the number of dyeing objects temporarily as 3(2-4). Suzuki (1963) reported that the number of dyed objects in homokaryon is 3 or 4 and in heterokaryon is 2,3,4 and 5 and

varies depending on the bacilli. It is very difficult to determine the number of dved objects in mycelial division but Shinkan observed six in P-2 species. Sometimes 5 nuclei are observed and this subject should be further studied. Also the existence of single and double nuclei can not be ignored.

IV Appearance of Variants on Culture Medium

A. Introduction

The nuclei in three cells of single spore isolates are considered to be genetically the same according to karyological studies through the living environment of bacilli. Even when the single spore isolates are cultured, the variation appears as the sectors and the variants contain entirely different bacilli from parental bacilli, (Konish 1933, Kuribayashi 1953, Shitayama et al 1964). We have clarified the status of variants appearance in potato gel culture media, employing many single spore isolates systems, and investigated the relationship between the appearance of variation and the number of nuclei.

B. Materials and methods

295 systems of single spore isolates from the various experimental stations in Japan between 1951-1953 are examined according to Kurozawa method (1955). Among these, 41 systems of mioga 13 systems, Hie 1 system, Kibi 1 system, Awa 7 system and Mehishiba 19 systems are the bacilli of plants other than rice.

The potato gel culture medium is placed in the container of diameter 8.5 cm and one bacilli clot from the single spore isolates are inoculated and incubated at 28°C in the dark. Five containers for one system are tested.

When sectors or island shape variants are appeared, an isolated culture is carried out on the inclined plane of potato gel medium and their characteristics are examined by three consecutive cultures in the same culture medium. These are classified into definite and temporary variants, and variants preserving the characteristics were treated as sectors. Also, the sectors which appeared in the process of three consecutive cultures were similarly cultured separately. The karyological studies are conducted in the same manner as previous experiments.

C. Results

1. Appearance of variants on potato gel culture medium and their characteristics.

295 systems of single spore isolates were investigated and the results on the frequency of sector appearance are shown in table 7. 245 systems among them (83.0 %) did not produce sectors and formed only homogeneous colonies. Average 3, 4 sectors for one container were the maximum. Saka-2, Saka-4, Saka-5, Saka-6 and Saka-7 were isolated from the same village (Hukushimaken Itatsukun) and considered to have a close relationship. The sectors appeared during the investigation as shown in table 8 were transplanted and their chacteristics were examined, in addition to the appearance of sectors. The results are shown in table 9. In most cases, the sectors do not appear after two times but in few cases, the sectors kept appearing even after two or three times transplantations.

Table 8. Appearance of sectors in single spore cultures on potato sucrose agar medium

No. of sectors per 5 plates	No. of sectors per plate	No. of strains		Remarks
0	0	245	(83%)	
1	0.2	20		
2	0.4	16		
3	0.6	6		
4	0.8	3	(17%)	75, 89-1-1, Saka-6
5	1.0	1		Saka-4
7	1.4	1		Saka-5
10	2.0	2		56, Saka-7
17	3.4	1		Saka-2
Total		295		

Table 9. Appearance of sectors in the course of subculture of sector forming strains on potato sucrose agar medium

No. of subcultures			No. of strains	Remarks
1st[1]	2nd[2]	3rd[2]		
1	0	0	13	
1	1	0	4	
1	1	1	1	
1	1	3	1	
1	1	4	1	
2	0	0	16	
3	0	0	6	
4	0	0	3	75, 89-1-1, Saka-6
5	2	0	1	Saka-4
7	3	3	1	Saka-5
10	2	0	1	Saka-7
10	4	1	1	56
17	19	25	1	Saka-2

1): No. of sectors per 5 plates.
2): No. of sectors appeared on slant cultures isolated from sectors formed on the first plate culture.

Figure 1 shows the process of consecutive culture of Saka-2 which shows the best sector appearance, and the culture properties are also noted. In some system,9 consecutive cultures still produced sectors and the variation of this system was very large. The variation of colony color was black, brown, yellow and grey.

2. Variation in the number of nuclei of the system

As shown in table 8 and 9, Saka groups such as Saka-2 showed many sectors and were very susceptible to variation. The ratio of multinuclei mycelial cells was low compared with other systems. Since some relationship may exist between these, number of nuclei in mycelial cells were examined on seventh culture of Saka-2, Saka-5 and 56. The results are shown in table 10. No significant difference is observed between 56 and system with 2.4 % multinuclei cell and sector appearance. Saka-2 and Saka-5 were clearly different from the original system. Particularly in Saka-2, the ratio of multinuclei cells varied from 1.3 % to 23.9 %, whereas the original system had 13.2%. Employing Saka-2, a similar investigations are conducted on 8 th and 9th culture and the results on the stability of their characteristics are shown in table 11 and Figure 1. There were species stabilized at high ratio of multinuclei cells (Saka-2-38, Saka-2-Z, Saka-2-57, Saka-2-36) and stabilized at low ratio of multinuclear cells (Saka-2-60, Saka-2-37, Saka-2-86) and unstable species.

Table 10. Variation in the number of nuclei in mycelial cells of variants appeared as sectors from Saka-2, Saka-5 and 56.

Strains	No. of nuclei in mycelial cells						No. of cells observed	No. of cells containing more than two nuclei	% of cells containing more than two nuclei	Average number of nuclei per cell
	1	2	3	4	5	6				
Saka-2	509	61	13	4			587	78	13.2	1.17
-X	401	105	9	10	1	1	527	126	23.9	1.31
-58	409	106	12	10		1	538	129	23.9	1.31
-9	404	94	6	5	1		510	106	20.7	1.25
-73	426	99	7	1	1		534	108	20.2	1.24
-72	441	91	4	2	1		539	98	18.2	1.20
-4-2	480	80	12	4			576	96	16.6	1.20
-8	441	70	3	5			519	78	15.0	1.19
-2-3-2	450	51	12	5			518	68	11.1	1.17
-Y	450	65	7				522	72	13.0	1.15
-38	463	59	5	3			530	67	12.6	1.15
-39	501	64	6	1			572	71	12.4	1.14
-37	469	63	2	1			535	66	12.3	1.13
-70	332	39	5				376	44	11.7	1.13
-4	501	47	5	3			556	55	9.9	1.12
-Z	485	53	3	1			542	57	10.5	1.11
-36	495	52	4				551	56	10.1	1.11
-1-7	477	49	4				530	53	10.0	1.11
-4-1	477	48	3	1			529	52	9.8	1.11
-37	530	28					558	28	5.0	1.05
-60	500	25	1				526	26	4.9	1.05
-78	552	27					579	27	4.6	1.05
-86	500	19	1	1			521	21	4.0	1.05
-25	339	14					353	14	3.9	1.04
-59	507	17					524	17	3.2	1.03
-40	502	16					518	16	3.0	1.03
-52	377	8					385	8	2.0	1.02
-41	522	10					532	10	1.8	1.02
-66	517	7					526	7	1.3	1.01
Saka-5	527	14					541	14	2.5	1.05
-1	503	64	3	1			571	68	11.9	1.24
-5	512	54	6	3	1	1	577	65	11.2	1.23
-17	503	46	2				551	48	8.7	1.18
-22	505	43	2	1			551	46	8.3	1.17
-11	505	29					534	29	5.4	1.11
-2	524	21	1	1			547	23	4.2	1.08
-10	500	18		1			519	19	3.6	1.07
-1-2	521	11					532	11	2.0	1.04
-0	534	10					544	10	1.8	1.04
56	520	13					533	13	2.4	1.02
-10	518	20					538	20	3.7	1.07
-0	534	19					553	19	3.5	1.06
-19	519	17					536	17	3.1	1.06
-14	519	17					536	17	3.1	1.06
-15	551	16					567	16	2.8	1.06
-8	508	13					521	13	2.4	1.04
-9	497	10					507	10	1.9	1.04
-16	511	8	1				520	9	1.7	1.03

Observation was made after seven subcultures on potato sucrose agar medium.

Table 11. Number of nuclei in mycelial cell at 7th~9th subculture of variants isolated from sectors of Saka-2

Strain	No. of subcultures	No. of nuclei in mycelial cell						No. of cells observed	No. of cells containing more than two nuclei	% of cells containing more than two nuclei	Average number of nuclei per cell	Significance of difference
		1	2	3	4	5	6					
Saka-2												
-38	7	463	59	5	3			530	67	12.6	1.15	
	8	501	56	2	2			561	60	10.6	1.12	
	9	508	53	3	1			565	57	10.0	1.11	
-72	7	441	91	4	2	1		539	98	18.2	1.18	***, ** ; *
	8	535	54					589	54	9.1	1.09	
	9	525	81	4	1			611	86	14.0	1.15	
-39	7	507	17					524	17	3.2	1.03	***
	9	553	58	5	1			617	64	10.4	1.12	
-73	7	426	99	7	1	1		534	108	20.2	1.22	*** ; ***
	8	513	55	5	2			575	62	10.7	1.12	
	9	496	47	1	1			545	49	9.0	1.10	
-X	7	401	105	9	10	1	1	527	126	23.9	1.31	*** ; ***
	8	500	64					564	64	11.3	1.11	
	9	478	77	1				556	78	14.0	1.14	
-60	7	500	25	1				526	26	4.9	1.05	
	9	520	27	1				548	28	5.1	1.05	
-59	7	501	64	6	1			572	71	12.4	1.14	*** ; ***
	8	516	61	6	1			584	68	11.6	1.13	
	9	530	31	1	1			563	33	5.8	1.06	
-Y	7	450	65	7				522	72	13.0	1.15	* ; *
	8	168	21					189	21	11.1	1.11	
	9	437	86	7	2	1		533	96	18.0	1.21	
-40	7	502	16					518	16	3.0	1.03	**
	9	524	39	3				566	42	7.4	1.07	
-41	7	522	10					532	10	1.8	1.02	***
	9	574	34	2				610	36	5.9	1.13	
-Z	7	485	53	3	1			542	57	10.5	1.11	
	8	479	50	5	2			536	57	10.6	1.12	
	9	509	55	3	1			568	59	10.3	1.09	
-57	7	469	63	2	1			535	66	12.3	1.13	
	8	494	79	4				578	84	14.5	1.16	
	9	510	63	8			1	581	71	12.2	1.14	
-36	7	495	52	4				551	56	10.1	1.11	
	8	509	48	6	1			564	55	9.7	1.11	
	9	517	55	2	2			576	59	10.2	1.11	
-37	7	530	28					558	28	5.0	1.05	
	9	515	31	2				548	33	6.0	1.06	
-70	7	332	39	5				376	44	11.7	1.13	*** ; ***
	8	461	109	13	7			590	129	21.8	1.26	
	9	524	122	9	4	4		661	137	20.7	1.26	
-38	7	409	106	12	10		1	538	129	23.9	1.31	* ; *
	8	502	100	10	7			619	117	18.9	1.23	
	9	452	126	13	2			593	141	23.9	1.27	
-66	7	500	19	1	1			521	21	4.0	1.05	
	9	513	24					538	25	4.6	1.05	
-17	9	531	69	5	3			608	77	12.6	1.14	
-4-1	7	477	48	3	1			529	52	9.8	1.11	
	9	511	64					575	64	11.1	1.11	

*: 5% level of significance
**: 1% level of significance
***: 0.1% level of significance

Table 12. Relationship between cultural conditions and number of nuclei in mycelial cell

Strain	Media*	No. of nuclei in mycelial cell					No. of cells observed	No. of cells containing more than two nuclei	% of cells containing more than two nuclei	Average number of nuclei per cell	Significance of difference
		1	2	3	4	5					
Saka-2-39	PAM	505	28	1			534	29	5.4	1.06	
	RAM	—	—	—			—	—	—	—	not
	SAM	506	40	3			549	43	7.8	1.08	
-80	PAM	536	26				561	26	4.6	1.05	
	RAM	501	20				521	20	3.8	1.04	not
	SAM	506	19				525	19	3.6	1.03	
-86	PAM	519	26				545	26	4.7	1.05	
	RAM	501	22				523	22	4.2	1.04	not
	SAM	530	15				545	15	2.7	1.03	
-41	PAM	500	30	7		1	538	38	7.0	1.09	
	RAM	513	40		1		554	41	7.4	1.08	not
	SAM	504	36	1			541	37	6.8	1.07	
-40	PAM	495	27				522	27	5.1	1.05	
	RAM	—	—	—			—	—	—	—	not
	SAM	520	15	1			536	16	2.9	1.03	
-81	PAM	540	22	1			563	23	4.1	1.04	
	RAM	564	16				580	16	2.7	1.03	not
	SAM	552	11	1			564	12	2.1	1.02	

* PAM: Potato sucrose agar medium
RAM: Rice straw decoction agar medium
SAM: Synthetic agar medium

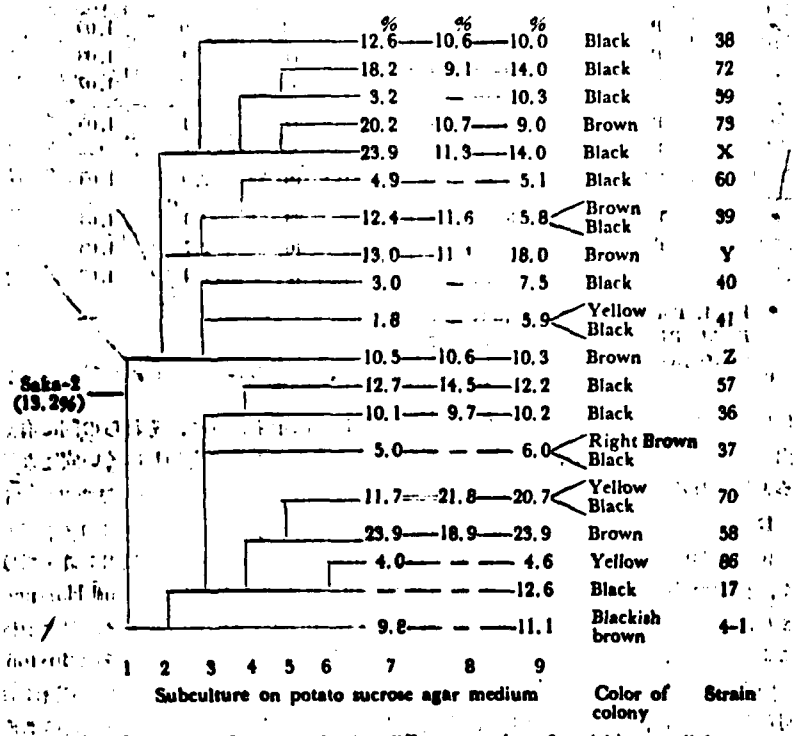

Fig. 1. Occurence of variants having different number of nuclei in mycelial cells of Saka-2.

Numerals show percentages of mycelial cells containing more than two nuclei.

: Difference is not significant

: Difference is significant

In order to investigate environmental effect on the number of nuclei in mycelial cells, the number of nuclei cultured in different composition of media such as potato gel, agaragar and synthetic gel were examined. The results are shown in table 12. No significant difference between them was observed. Thus, the number of nuclei per cell is not affected by environmental changes and considered to be fixed.

D. Discussion

A sudden variation in culture properties occurs in bacilli and its frequency of occurrence depends on its kind and biotype (Stakman and Harrar 1957). The appearance of different shapes of sectors in colony is due to either nuclear isolation in heterokaryon or sudden variation in homokaryon

(Buxton 1961). The variations in piricularia species appear occasionally on culture media and as is clear from the above experiment, the appearance of sectors and its frequancy are considerably different depending on the systems. It is noticed that there was no relation between the number of nuclei in a cell and the frequency of sector appearance. In most systems, multinuclear cells are very low and the sector appearances are almost not noticed. In system where the sector appearances are many, there are species with relatively high ratio of multinuclear cells such as Saka-1 and Saka-2 and species with relatively small ratio of multinuclear cells such as Saka-5 and 56.

Since the nuclei in conidial cells are genetically the same, the single spore isolates can be considered as pure. Therefore, the frequent sector appearances were considered to be due to the following.1) A sudden variation appears in growth process. 2) Heterokaryon is isolated due to anastomosis between the variants which appeared in the same culture system. 3) It may be due to parasexual recombination of already existing or newly created hetero double nuclei. We can not conclude on the causes of sector appearance from these experimental results but an inferrence can be made that the possibility of 1) and 2) is large for Saka-2 which has the high frequency multinuclei cell appearance and the possibility of 1) and 3) is large for Saka-5 and 56 which have low frequehcy of multinuclei cell appearance. Further investigations are necessary on this subject for satisfactory answer.

When the sector is isolated and cultured consecutively, the systems different from multinuclei cells are isolated. In Saka-2 with high frequency of multinuclei cells, systems from maximum 23.9 % multinuclei cells of Saka-2-X to minimum 1.3 % of Saka-2-66 are isolated by seven consecutive culture. In Saka-5 with low frequency of multinuclei cells appearance, the variation is seen from 1.8 % to 11.9 %. In 56, the variation is only 1.8 % to 11.9 %. In 56, the variation is only 1.7 % to 3.7 %. These differences between the systems change sometimes but 9 consecutive cultures seem to stabilize them. Thus, the cell with different average number of nuclei can be obtained by isolating the variants, but the reasons for these have to be clarified by future investigation. As shown in table 12, the change in the number of nuclei due to environment is very difficult. Therefore, the multinuclei cells (average number of nuclei per cell) are considered to be genetically stable against environmental changes.

V. Anastomosis and Parasexual Recombination

A. Introduction

Since sexual generation is not yet discovered in incomplete bacilli, the causes for variations are considered to be due to 1) sudden variation and 2) recombination of nuclei with different genetic type by heterokaryos. In 1952, Ponteconvo and Roper indicated the existence of parasexual cycle in Aspergillus nidulans and the variation due to somatic recombination of genetic elements in addition to the possibility of genetic analysis. The research in these areas has remarkably progressed ever since. Tody, the existence of parasexual cycles is established for the following. Aspergillus niger (Pontecorvo et al, 1953), Penicillium chrysogenum (Pontecorvo and Sermonti, 1954), Fusarium f. pisi (Buxton 1956), Aspergillus oryzae and Aspergillus soyae Oshitani et al, 1956), Cephalosporium mycophilum (Tuveson and Coy 1961), Cochliobolus sativus (Tinline 1962), Verticillium albo-atrum (Hastie, 1962), Fusarium oxysporum f. Cubense (Buxton, 1962) and Penicillium expansum (Barron, 1962)

It is very important to determine the existance of parasexual cycle in bacilli and establish the causes of variations in somatic recombination. The following are the preliminary results of our experiments and reported here since we thought that somatic recombination can be obtained in rice blast fungi.

B. Materials and methods

Observation of nuclear activity in anastmosis:

Employing system 58-4 susceptible to anastomosis, the culture is carried out on slide glass in straw gel liquid media for 30 hours at 23°C. Phenomenon of anastmosis is observed with microscope and the dyeing of nuclei is conducted by HCl-Giemsa method.

Method of inducing anastmosis:

Variants such as P-2b-XN42-XI*, P-2b-XN98*, P-2b-XN106br*, Ken 53-33-Cu9 and S225 obtained from three different system i,e, P-2b, Ken 53-33 and S225 by X-ray, ultraviolet light treatment and CuSO4 potato gel culture were employed. The nutritional requirements and morphological properties are shown in table 13.

(* Obtained by Yamazaki and Murada (1963)).

Table 13. Strains used for the experiments of anastomosis and parasexual recombination

Strain	Nutrient requirement					Generation of H_2S	$CuSO_4$ resistance	Character of colony on potato sucrose agar medium			Remarks
	Adenine	Cystine or methionine	Inositol	Lysine	Phenylalanine			Color	Aerial hyphae	Growth	
P-2b	+	+	+	+	+	−	−	black	++	normal	
Ken 53-33	+	+	+	+	+	−	+	black	++	normal	Isolated from wild strain
S225	+	+	−	+	+	−	+	black	++	normal	
P-2b-XN 42X1	−	+	+	−	+	−	−	black	±	normal	X-ray treatment
P-2b-XN 98	−	+	+	+	+	−	−	black	±	normal	〃
P-2b-XN 106 br	+	+	+	+	−	−	−	brown	++	dwarf	〃
Ken 53-33-Cu 9	+	−	+	+	+	+	+	black	±	normal	$CuSO_4$ treatment
S 225-107	+	−	−	+	+	+	−	pale black	++	normal	〃

+: No requirement of nutrient, generation of H_2S and resistance to $CuSO_4$.
−: Requirement of nutrient, no generation of H_2S and no resistance to $CuSO_4$.

The following three methods were employed in inducing anastomosis. 1) Conidium and mycelium from potato gel culture were placed in sterilized water and the solutions were prepared by mixing various amounts of two systems. These were centrifuged to make a mass of conidium and mycelium. These were cultured in a small amount of agar medium fro 2-3 weeks at 28°C. Then, the active part appears as sector. This part is again transplanted to agar culture medium. 2) The systems for experimental use were separately cultured fro three weeks at 28°C on potato liquid culture medium in which few pieces of cotten cut in about 5 cm length were placed. The bacilli adhered cotten was transferred to a small amount of agar culture medium and two systems were cultured side by side. Then, the active growth part appears from the mixed colony on agar culture medium. This is again transplanted to a small amount of agar culture medium. 3) A mixture of two systems was cultured on potato liquid medium. After two weeks, the colonies were washed four times with sterilized distilled water and cut with platinum wire. The piece is inoculated on a small agar culture medium. After 7 days of culture, if the colonies appear, these are transplanted to small culture medium. The transplantation of these colonies on small agar culture medium was repeated 2-3 times and the characteristics of growth and colony were karyologically investigated.

C. Results

1. Nuclear activities in anastmasis.

The number of nuclei in anastomosis of micelia and the locations were observed on 423 cells, and the results are shown in table 14 and figure plate II. A nucleus exists separately in anastomosis of both mycelial cells. (table 14A, figure plate II-A, 1) and this is most abundant (77 %). Also the following are observed. One nucleus in anastomosis of mycelia is about to migrate into the other cell through anastomosing part. (table 14 B, figure plate II-A, 2). No nucleus exists in one cell and two nuclei are included in the other cell (table 14 C, figure plate II-A, 3). The nuclei in both cells exist very close together at anastomosing location of mycelia, or two nuclei are partly anastomosed. (table 14 D, figure plate II-A, 4a, figure plate II-B, 4b). Only one nucleus is observed through both anastomosing part or anastomsis of mycelia (table 14 E, figure plate II-B, 5a, 5b, 5c). And table 14, F, G are observed.

Table 14. Behaviour of nuclei in anastomosis of mycelia of strain 58-4

Type	No. of cells observed	%
A	326	77.0
B	21	4.9
C	5	1.2
D or	15	3.6
E or	48	11.4
F	1	0.2
G	7	1.7
Total	423	100.0

Observation was made by staining nuclei with HCl-Giemsa method after 30 hours culture in the rice straw decoction liquid medium.

/: The point at which anastomosis occured.

From the above observation, it is certain that a nucleus of one cell is migrated into the other cell through anastomosing part of mycelia. The migrated nucleus can possibly be anastomosed with the nucleus in the other cell and forms a nucleus of twice the size. However, this should be confirmed by measurement of DNA, determination of the number of chromosome and other method. At any rate the bacilli from anastomsis of mycelia are considered to be either heterocaryon which is used together with nucleus of biochemical variants, or heterozygous diploid from anastomosis of both nuclei. Since karyological observations indicate that one cell contains one nucleus mostly, the possibility of latter is high. In this paper, we combine both and designate as heterodiploid.

2. Properties of heterodiploid

Before the mixtures of biochemical sudden variants are cultured, the high concentrations of conidium and mycelia were inoculated on small agar culture medium in order to investigate whether they grow by reversion of sudden variation. When occurrance of cross feeding is examined by culture of two sudden variants on agar culture medium the variants employed in these experiments did not grow on culture media and no cross feeding nor the reverse sudden variation occurred.

Employing six biochemical variants, attempt was made to form diploid by various combination between them. The combinations showing active growth similar to wild bacilli are shown in Table 15. These maintained their characteristics in culture by transplantation of mycelial mass. Among these culture mixtures, for the case of one system producing H_2S gas (II, III, IV), or even for both systems producing H_2S gas (V), no H_2S gas production is observed.

Table 15. Character of newly formed heterodiploid

Hetero-diploid	Combination	Nutrient requirement					Gene-ration of H_2S	$CuSO_4$ resis-tance	Character of colony on potato sucrose agar medium		
		Ade-nine	Cystine or Me-thionine	Ino-sitol	Lysine	Phenyl-alanine			Color	Aerial hyphae	Growth
Het I	P-2b-XN 98+ S 225	+	+	+	+	+	−	+	black	++	normal
Het II	P-2b-XN 98+ S 225-107	+	+	+	+	+	−	−	black	++	normal
Het III	P-2b-XN 98+ Ken53-33-Cu9	+	+	+	+	+	−	+	black	++	normal
Het IV	S 225-107+P-2b-XN 42X1	+	+	+	+	+	−	−	black	++	normal
Het V	S 225-107+ Ken53-33-Cu9	+	+	+	+	+	−	+	black	+	normal
Het VI	P-2b-XN 98+ P-2b-XN106br	+	+	+	+	+	−	−	black	+	normal

Symbols are the same with Table 13.

When the resistant bacilli are employed for the combination of culture (I,IV,V), They showed $CuSO_4$ resistance. When colony on potato agar medium is brown (VI), heterodiploid was black and normal.

3. Recombination obtained by single spore isolation of heterodiploid.

Conidium formed on heterodiploid on small culture medium was inoculated on potato agar medium and nutrient requirement and the properties of colony were examined by taking the colony formed from single spore. The results are shown in table 16.

Het-I is heterodiploid formed by P-2b-X'98 (Adenine required, no. H_2S generation and sensitive to $CuSO_4$) and S225 (insitol required, H_2S is generated, $CuSO_4$ resistant), does not possess nutrient requirement, is $CuSO_4$ resistant and does not generate H_2S. 160 single spore isolates were investigated and found the following. 31 systems were investigated and found the following. 31 systems had the same properties as S225 except the lighter color of colony. 15 systems had the same properties as Het-I. One system had grey color of colony and one system had grey color of colony but the adenine requirement and $CuSO_4$ sensitivity are the same as P-2b-X'98. Two systems had inositol requirement, $CuSO_4$ sensitivity and brown colony color. It is particularly significant that 110 systems (69%) with cystine or methionine requirement and brown colony color entirely different from parental system appeared.

Het-II is heterodiploid formed by both system of P-2b-X'98 and S225-107 (Cystine or methionine and inositol requirement, H_2S generation and $CuSO_4$ resistant), which does not show nutrient requirement, generates H_2S gas and is suceptible to $CuSO_4$. When 120 single spore isolates were investigated, the following systems were obtained. Two systems had the same properties as Het-II. Systems having cystine or methionine requirement, H_2S generation and $CuSO_4$ suceptibility are 16 for black colony color, 72 for brown colony color and 8 for white colony color respectively. 22 systems had adenine, cystine or methionine, inositol and other materials requirements, no H_2S generation and $CuSO_4$ suceptibility.

Het-III is heterodiploid formed by both systems of *2b-X'98 and Ken 53-33-Cu9 (Cystine or methionine required, H_2S generated, $CuSO_4$ resistant), which does not show nutrient requirements, is resistant to $CuSO_4$ and does not generated H_2S. Among 119 single spore isolates, 35 systems had exactly the same propepties as Ken 53-33-Cu9 and 9 systems had the same

properties as Ken 53-33-Cu9 except less H_2S generation. Other systems show recombination between both parental systems concerning nutrient requirements, H_2S generation and $CuSO_4$ resistance and the color of colonies was entirely different. It is significant that system having adenine requirement did not appear.

Het-IV is heterodiploid formed by both systems of P-2b-X'42-XI(adenine and lysine required, no H_2S generation, susceptibel to $CuSO_4$) and S225-a07, which does not have nutrient requirement, does not generate H_2S and is susceptible to $CuSO_4$. Among 149 single spore isolates, 45 systems had the same properties as P--2b-X'42-XI. 10 systems had the same nutrient requirement and H_2S generation as S225-107 but had different color of colony and growth from both parental systems. other systems showed the recombination type on the nutrient requirements.

In summarizing above results, heterodiploid shows the following properties. 1) No nutrient requirements. 2) No H_2S generation 3) Appearance of resistance when both parental systems are different in $CuSO_4$ resistant. In the case of single spore isolation, 1) some systems show the same properties as parental systems (Het-III and IV) but most systems show different properties from parental systems. 2) Het-I, II and III require cystine or methionine and many systems showed brown color of colony. It is significant that the different properties from parental systems appeared in Het-I. 3) Appearance of the systems having the same properties as heterodiploid are very rare. These phenomena do not appear due to isolation of nuclei in heterocaryon.

D. Discussions

When two kinds of biochemical sudden variants with different nutient requirements were cultured, the appearance of bacilli on small culture medium is considered to be due to one of the following. 1) Formation of heterocaryon. 2) Simple mixed bacilli. 3) Cross feeding or 4) Reverse sudden variation. Since the separate experiment showed that reverse sudden variation and cross feeding do not occur in the blast fungi, the appearance of bacilli on small culture medium is considered to be due to former two. As is clear from this experiment, the nucleus in one cell migrates into the other according to anastomosis of mycelia. The formation of heterocaryon might be the main cause for this phenomenon. However, one nucleus in one cell is a rule in bacilli and the migrated nucleus combine with the other nucleus in the cell to form one nucleus according to this experimental observations. Therefore, it is

Table 16. Characters of single spore cultures isolated from heterodiploid

Heterodiploid and their parental strains	Nutritional requirement				Generation of H_2S	$CuSO_4$ resistance	Character of colony			No. of single spore cultures	Remarks
	Adenine	Cystine or methionine	Inositol	Lysine			Color	Aerial hyphae	Growth		
P-2b-XN 98	−	+	+	+	−	−	black	±	normal		
S 225	+	+	−	+	−	+	black	++	normal		
Het-I	+	+	+	+	−	+	black	++	normal		
Single spore cultures	+	+	−	+	−	+	pale black	++	normal	31	
	+	−	+	+	−	−	black	+	normal	110	
	+	+	+	+	−	+	brown	++	normal	15	Het-I type
	+	+	−	+	−	−	blackish brown	+	normal	2	
	+	+	−	+	−	+	gray	+	normal	1	
	−	+	+	+	−	−	gray	+	normal	1	
Total										160	
P-2b-XN 98	−	+	+	+	−	−	black	±	normal		
S 225-107	+	−	−	+	+	−	pale black	++	normal		
Het-II	+	+	+	+	−	−	black	++	normal		
Single spore cultures	−	−	−	?	−	−	black	±	normal	22	
	+	−	+	+	−	−	black	++	normal	16	
	+	+	+	+	−	−	black	++	normal	2	Het-II type
	+	−	+	+	−	−	brown	+	normal	72	
	+	−	+	+	−	−	white	±	normal	8	
Total										120	

P-2b-XN 98	−	+	+	+	−	−	black	±	normal		
Ken 53-33-Cu 9	+	−	+	+	+	+	black	±	normal		
Het-III	+	+	+	+	−	+	black	++	normal		
	+	−	+	+	+	+	black	±	normal	35	Ken 53-33-Cu 9 type
Single	+	+	+	+	+	+	black	+	normal	4	
spore	+	−	+	+	−	−	brown	+	normal	65	
cultures	+	−	+	+	−	−	white	±	normal	5	
	+	−	+	+	±	−	white	±	normal	1	
	+	−	+	+	±	+	black	±	normal	9	
Total										119	
S 225-107	+	−	−	+	+	−	black	++	normal		
P-2b-XN 42-X 1	−	+	+	−	−	−	black	±	normal		
Het-IV	+	+	+	+	−	−	black	++	normal		
	+	−	−	+	+	−	black	++	normal	46	S 225-107 type
Single	−	+	+	−	−	−	black	±	normal	26	P-2b-XN 42-X1 type
spore	+	+	−	+	+	−	pale black	+	normal	65	
cultures	+	−	+	+	+	−	black	+	normal	2	
	+	−	−	+	+	−	blackish brown	±	dwarf	10	
Total										149	

concluded that the systems growing on small culture medium contain one nucleus of heterodiploid in one cell.

Thus, the bacilli are self-nutrient, normal H_2S generation in any combination and shows superior $CuSO_4$ resistant. In single conidial isolates, very few had the same properties as parental strain or heterodiploid concerning the shape and color of colony and most of them were considered to have recombination of various characteristics. It is significant that strains requiring cystine or methionine were like their parental strain as in the case of Het-I were isolated. In other cases, the strains whose color of colony is different of parental strain were isolated.

Pantecorvo and Roper (1952) proved the existence of recombination cycle in Aspergillus nidulans and designated this as parasexual cycle (Pontecorvo 1956). These cycles have the following 5 steps. a) Two different nuclei in heterocarvon fuse into one. b) As a result, heterodiploid nucleus is formed and multiply. c) Diploid bacilli like homocarvon are formed as a strain. d) Cell transfer is occurring during multiplication of diploid nuclei. e) Diploid nuclei generate a single phase nutrient cell.

In order to prove the existence of parasexual cycle in bacilli, it is necessary to establish each steps described above. As mentioned previously, migration and fusion of nuclei occur along with anastomosis of mycelia and 11.4 % contain only one nucleus in two cells between which anastomosis occurred. These strongly support the step a). A direct proof on step b) and c) was not obtained but the appearances of many recombinations in single-conidial isolates strains formed by self-nutrient and two different biochemical variants support the phenomena of step d) and e).

Mechanism on appearances of nutrient requirements and colony color which were not possessed by parental strains is not clear but can be speculated to be due to transfer between structure and adjustment hereditary elements.

At any rate, the above facts suggest the existence of parasexual cycle and recombination and transfer of cells may be a cause for appearance of variation.

It will be necessary in the future to establish 1) existence of nucleus and diploid nucleus by determining amount of nucleic acid and number of chromosome and 2) existence of strain having diploid nuclei like homocarvon. It is also necessary to establish these phenomena by 3) analyzing the recombination and transfer in detail.

The part of the results on single spore isolates strains above do not disprove the possibility of nuclei isolation in simple heterocaryon.

VI Summary

I. Karyological studies of Piricularia oryzae, the pathogen of rice blast, and other Piricularia species infectious to plants other than rice were conducted from the point of view to clarify make clear the genetic mechanisms of variability in these organisms.

(1) Results of investigations carried out on 28 strains of Piricularia oryzae are as follows:

(a) Among nuclear straining procedures tried, those with Giemsa's solution, basic fuchsin, aceto-orcein, or aceto-carmine proved to be suitable for the purpose, whereas those with azure A or haematoxylin were not suitable.

(b) Majority of cells in conidia, germ tubes, mycelia, and conidiophores were found to be uninuclear. Cells containing two or more nuclei were rarely observed.

(c) In germinating conidium, the nucleus migrates into germ tubes in either of the following two ways. In one, the nucleus of conidial cell divides, and one of the divided halves migrates into the germ tube. In the other, the nucleus migrates into the germ tube without division.

(d) In the process of conidium formation, the nucleus of the apical cell of conidiophore divides and one of the divided nuclei migrates into the newly formed unicellular conidium, and the nucleus of the conidium divides again and bicellular conidium is produced by forming septum between the two divided nuclei. The nucleus of either cell of the bicellular conidium divides and the tricellular conidium each cell of which contains one nucleus is finally formed. It was deduced from the above observation that the nuclei contained in the three cells of a conidium are uniform in their genetic constitution, and accordingly, a strain derived from a single spore is genetically homogeneous.

(e) The nuclear division observed in the cell of conidium during its germination is typical mitosis, the chromosome number counted at metaphase and anaphase being 5-6 or about 3. Detail of the nuclear division in the mycelium is still obscure. It is not considered to be a typical mitosis.

(2) Piricularia species isolated from Setaria italica, Panicum Crus-galli, Panicum miliaceum, Digitaria adscendens and Zingiber Mioga were karyologically investigated. Uninuclear cells predominated also in mycelia of these organisms.

II. Comparison was made of frequencies of sectoring of 295 strains derived from single-spore isolates of Piricularia oryzae, on potato sucrose agar medium. In some of the strains, relationship between the frequency of sectoring and the number of nuclei in a cell, and the shift of the nuclear number with sectoring were investigated.

(a) Sectors appeared in 17 per cent of the strains tested. Some strains which produced sectors at extraordinary high frequencies were found, and among them Saka-2, Saka-5, and 56 were examined for nuclear number in mycelial cells. Saka-5 and 56 were found to consist almost entirely of uninuclear cell. In Saka-2, on the contrary, considerable proportion (ca. 13.2%) of the cells contained 2-4 nuclei. The variants appeared as sectors were diverse in their nuclear numbers. Some included more than 20 per cent of the multinuclear (2-6 nuclei per cell), the percentage being stable. Some, on the other hand, showed the frequency of multinuclear cells being ca. 1.3 per cent. There was a tendency in Saka-2 and Saka-5 to produce variants in which the percentage of multinuclear cells was relatively high, whereas all the variants derived from 56 were predominantly uninuclear.

(b) The frequency of the multinuclear cells in any strain was hardly affected by environmental conditions (composition of the culture media).

(c) Findings described above strongly suggest that the number of nuclei in a cell is the trait to be determined genetically.

III. Nuclear behavior in anastomosis in Piricularia oryzae was investigated. By allowing two different auxotrophic strains marked also differently with H_2S generation and $CuSO_4$ resistance anastomosis, variants were obtained which can grow in the synthetic minimal medium. The characteristics of these variants and single-spore isolates from them were also investigated.

(a) In the course of anastomosis, the nucleus of a strain migrates into cell of another strain to produce a heterodicaryotic cell. In a number of the dicaryotic cells, two nuclei were observed to be closely located, and occasionally there were cases in which the two nuclei were thought to have eventually fused to become diploid.

(b) In diploids between two different auxotrophs, which are phenotypically prototrophic, $CuSO_4$-resistance appeared to be a dominant character while H_2S-generation was recessive in any strain tested.

(c) Among single-conidial isolates of heterodiploids, many showing recombinant characteristics in respect of auxotrophy, H_2S-generation, and $CuSO_4$-resistance were detected besides those of entirely parental characteristics and of the characteristics of heterodiploids.

(d) Single-spore isolates from heterodiploids frequently showed unexpected characteristics which were not posessed by any of parental strains.

(e) The above observations suggest that the parasexual recombination is possible in this organism.

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Explanation of Plates

Plate I-A

1 Nucleus in conidial cell.
2 Nucleus in conidial cell (four celled conidium, aceto-carmine staining).
3 Nucleus in conidial cell viewed under Phase Contrast Microscope.
4a - 5b Migration of nucleus to germ tube; 4a and 4b, migration of nucleus without nuclear division; 5a and 5b, migration of one nucleus after nuclear division.
6 and 7 Increase of the number of nuclei in conidium by nuclear division after germination; 6, apical-cell 2 nuclei, central-cell 4 nuclei, basal-cell 2 nuclei; 7, central-cell 8 nuclei.

Plate I-B

8 and 9 Nucleus in mycelial cell.
10 Nuclei in conidiophore and conidium at one cell stage.
11 - 12 b Nucleus in conidial cell at one cell stage; 12b, viewed under Phase Contrast Microscope.
13a and 13b Nuclei in conidial cell after the first nuclear division in conidium.
14 Nucleus in conidial cell at two cell stage.

Plate I-C

15 Nuclear division in apical-cell of conidium at two cell stage.
16 Nucleus in appressorium.
17 - 23 Nuclear division in conidia; 17, late prophase (aceto-orcein staining); 18, late prophase (view under Phase Contrast Microscope); 19, metaphase; 20 and 21, anaphase; 22 and 23, telophase.

Plate I-D

24 and 25 Chromosomes in anaphase of conidial nuclear division (aceto-orcein staining); About 6 chromosomes are observed.
26 - 31 Nuclear division in mycelial cell; Chromatin masses connected in irregular rosary form split longitudinally (27) and separate into two groups (28). These two groups move to the opposite direction along the length of mycelial cell.

Plate II-A

1 One nucleus is observed in each of the cells between which anastomosis occurred.
2 Nucleus in one of the cells between which anastomosis occurred is moving to the other.
3 Two nuclei are observed in one of the cells between which anastomosis occurred, but in the other none.

4a and 4b Nuclei of both cells between which anastomosis occurred are closely located to each other or fuse at the point of anastomosis.

Plate II - B

5a - 5c Only one nucleus is observed in both cells between which anastomosis occurred.